Taurus Energy AB

Attachment 8

THIS DOCUMENT CONTAINS CONFIDENTIAL BUSINESS INFORMATION

Antonie van Leeuwenhoek **61**: 249–257, 1992.

The value of electrophoretic fingerprinting and karyotyping in wine yeast breeding programmes

T.J. van der Westhuizen & I.S. Pretorius*
Department of Microbiology and Institute for Biotechnology, University of Stellenbosch, Stellenbosch 7600, South Africa (request for offprints)*

Accepted in revised form 29 October 1991

Key words: wine yeasts, fingerprinting, CHEF, karyotyping, spore-cell mating

Abstract

Electrophoretic banding pattens of total soluble cell proteins, DNA restriction fragments and chromosomal DNA were used to characterise ten strains of *Saccharomyces cerevisiae* used for commercial production of wine. These fingerprinting procedures provided unique profiles for all the different yeast strains and can therefore be used to identify and control industrial strains. Furthermore, the protein profiles, restriction fragments banding patterns and electrophoretic karyotyping by contour clamped homogeneous electric field electrophoresis (CHEF), were valuable to differentiate hybrid and parental strains in yeast breeding programmes. Hybrid strains, with desirable oenological properties, were obtained by mass spore-cell mating between a heterothallic killer yeast and two homothallic sensitive strains and all were shown to have unique DNA fingerprints and electrophoretic karyotypes.

Introduction

Unlike other yeast-based industries such as baking and brewing, the wine industry has not taken an active interest in yeast genetics and strain-development programmes (Thornton 1983). With traditional wine fermentation methods there was little need to manipulate the yeast strain. However, new trends in beverage markets demand the modification of traditional wine yeast strains and the development of more cost-effective winemaking practices. The fact that the requirements of the wine industry for yeast stocks have not been defined in genetic terms, has impeded the identification of realistic targets for strain development. Furthermore, genetic programming of homothallic wine yeasts by inter-strain hybridisation was problematical. This obstacle was, however, overcome by breeding techniques such as spheroplast fusion, rare mating and mass spore-cell mating (for a review see Pretorius & Van der Westhuizen 1991). The successful application of these genetic techniques in strain development depends on the ability to differentiate between parental and hybrid strains.

Yeast cultures used in the alcoholic beverage industries are usually characterised by cell and colony morphology, physiological test and the ability to flocculate or to form a pellicle (Kunkee & Amerine 1970). However, these techniques are not universally adept at differentiating between strains of the same species. Furthermore, it is apparent that many of the physiological and biochemical characteristics used for identification are encoded by a small portion of the genome. This resulted in the fingerprinting of industrial yeast strains by protein profiles (Van der Westhuizen & Pretorius 1989, 1990; Van Vuuren & Van der Meer 1987), re-

striction fragment length polymorphisms of genomic or mitochondrial DNA (Keiding 1985; Lee & Knudsen 1985; Panchal et al. 1987; Pedersen 1985b, 1986a), electrophoretic karyotyping (chromosomal banding patterns) (Casey & Pringle 1990; Petering et al. 1990; Van der Westhuizen & Pretorius 1989, 1990; Vezinhet et al. 1990) and gas-liquid chromotographic analysis of the cellular long-chain fatty acids (Augustyn & Kock 1989; Tredoux et al. 1987).

The present report describes the characterisation of ten wine yeast strains by visual comparison of total soluble cell protein patterns, restriction fragment banding patterns and electrophoretic karyotyping. We also describe the hybridisation of a heterothallic, killer yeast with two homothallic, sensitive strains by mass spore-cell mating. This report highlights the value of electrophoretic fingerprinting and karyotyping in breeding programmes.

Materials and methods

Yeast strains and genetic methods

The following strains of *Saccharomyces cerevisiae*, obtained from the Viticulture and Oenological Reseach Institute (VORI; Stellenbosch, South Africa), were used in this study: N6 (WE14) , N21 (Geisenheim), N66 (WE372), N76 (228), N91 (WE466), N93 (WE500), N95, N96, N97 and N181 (VIN7). Strains N93 and N181 originated from the same culture. Standard yeast genetic methods of sporulation, purifying and selecting haploids were carried out according to Sherman et al. (1986). Hybridisation between haploid cells isolated from heterothallic strains, and ascospores isolated from homothallic strains, was performed according to the mass spore-cell mating method described by Salmon et al. (1989).

Media and screening procedures

Yeast strains were grown in a complex medium (YPD) consisting of 1% yeast extract, 2% peptone and 2% glucose. Sporulation of diploid cells was induced in SP medium containing 1% potassium acetate, 0.1% yeast extract and 0.05% glucose. Galactose utilising strains were identified by the presence of yellow halos on YPGB medium containing 1% yeast extract, 2% peptone, 2% galactose and 2% bromothymol blue (4 mg/ml). Methylene blue agar plates, buffered at pH 4.5, were used to detect zones of growth inhibition caused by the K_2 killer toxin secreted by killer yeasts.

Isolation and electrophoresis of proteins

The inoculum size was standardized to obtain 10^4 cells/ml in 1 *l* YPD broth in 2 *l* Erlenmeyer flasks. Cultures were incubated at 30° C on a rotary shaker until the cell counts reached 10^8 cells per ml. Preparation of cell-free extracts and isolation of total soluble cell proteins from yeasts were carried out according to the methods described by Van Vuuren & Van der Meer (1987). Protein extracts were stored at − 18° C. The protein concentration was determined by the Folin-Lowry method (Plummer 1971) and samples were adjusted to a concentration of 2 mg/ml with 6.4 mM Tris (hydroxymethyl) aminomethane buffer (pH 8.4). Slab gel electrophoresis (SE 600 Cooled Vertical Slab Unit; Hoefer Scientific Instruments, San Francisco, USA) was used to obtain protein profiles. The lower electrode buffer [63 mM Tris (hydroxymethyl) aminomethane, 50 mM HCl, pH 7.5] was kept at 8° C. The upper electrode buffer contained 37.7 mM Tris (hydroxymethyl) aminomethane and 40 mM glycine (pH 8.9). The gel was 1.5 mm thick and consisted of a 5% (w/v) acrylamide stacking gel and a 7% (w/v) acrylamide resolving gel. Samples of 50 µl were loaded into the wells. Bromophenol blue (0.1%, w/v) in 50% (w/v) sucrose served as loading buffer. Electrophoresis was performed at a constant current of 35 mA for 5 h. The gels were fixed with 12.5% (v/v) trichloric acid, stained with 0.25% (w/v) Coomassie blue R-250 and destained with a 7% (v/v) acetic acid-5% (v/v) methanol solution.

Isolation of genomic DNA and electrophoresis of restriction fragments

Genomic DNA was isolated from the parental and hybrid strains according to a method reported by Gupta & Jones (1987). The DNA was digested with the *Hae*III restriction endonuclease according to the specifications of the supplier (Boehringer Mannheim Biochemicals, Mannheim, Germany). Electrophoresis of DNA samples was performed in a 0.8% (w/v) agarose gel in TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA, pH 8.0) at 100 V for 2 h. Gels were stained with ethidium bromide (10 mg/ml) and viewed on a transilluminator.

Preparation of intact chromosomal DNA and pulsed field gel electrophoresis

Chromosomal DNA samples were prepared according to the embedded-agarose procedure of Carle & Olsen (1985). Intact chromosomal DNAs were separated using contour clamped homogeneous electric field (CHEF) electrophorsis. The apparatus used was the CHEF-DR II (Bio-Rad Laboratories, Richmond, USA). All CHEF separations were carried out in a 20 cm square, 6 mm deep, 1.2% agarose gel made in 0.5 × TBE buffer. Thin sections of the DNA-agarose plugs were loaded into the wells and sealed in with 1% low melting temperature agarose just prior to the run. The average running temperature of the 0.5 × TBE electrophoresis buffer was maintained at 14° C by a recirculating water bath set at 4° C. Gels were run for 26 h at a constant voltage of 200 V. The pulse duration was 60 s for the first 15 h and 90 s for the last 11 h. Gels were stained with ethidium bromide (10 mg/ml) and viewed on a transilluminator.

Results

Fingerprinting of wine yeast strains

The electrophoretic banding patterns of total soluble cell proteins (Fig. 1), DNA restriction fragments (Fig. 2) and chromosomal DNA (Fig. 3) were used to characterise ten strains of *S. cerevisiae* used for commercial production of wine. Variation of the profiles of strains N6, N21, N66, N76, N95 and N97 were apparent in the number, position and intensity of the bands. Strains N93 and N181 originated from the same culture and, as expected, displayed similar characteristic protein profiles, however, the DNA restriction fragment and chromosomal banding patterns differed slightly. Similar protein banding patterns and DNA profiles (data not shown) were obtained for strains N93′ (a strain once thought to be synonymous with strain N93) and N95, supporting the hypothesis that they originated from the same culture. Identical profiles were also obtained for killer strain N96 and strain N91. Strain N91 is a derivative of strain N96, cured of the K_2 killer character. Although, similar protein

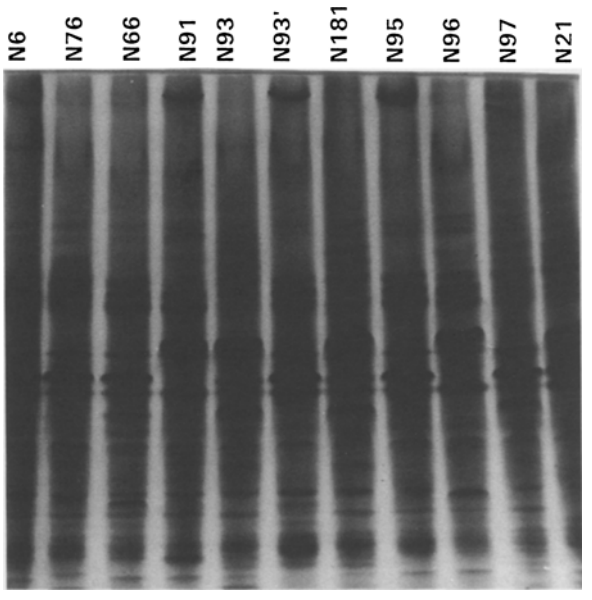

Fig. 1. Tota soluble cell protein patterns of wine yeast strains N6, N76, N66, N91, N93, N93′, N181, N95, N96, N97 and N21. Strains N93 and N181 originated from the same culture. Strain N91, a derivative of N96, is cured of the killer character. Strain N93′ was previously mistakingly distributed as N93, but was also later shown to be N95. Electrophoresis was performed in a 5% (w/v) acrylamide stacking gel and a 7% (w/v) acrylamide resolving gel.

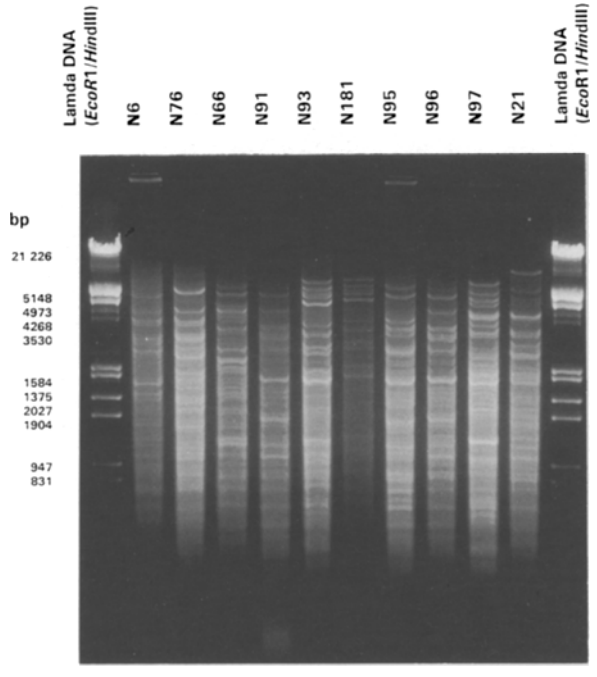

Fig. 2. DNA restriction fragment banding patterns of wine yeast strains N6, N76, N66, N91, N93, N181, N95, N96, N97 and N21. Total genomic DNA of these strains was cleaved with *Hae*III and separated in a 0.8% agarose gel, stained with ethidium bromide.

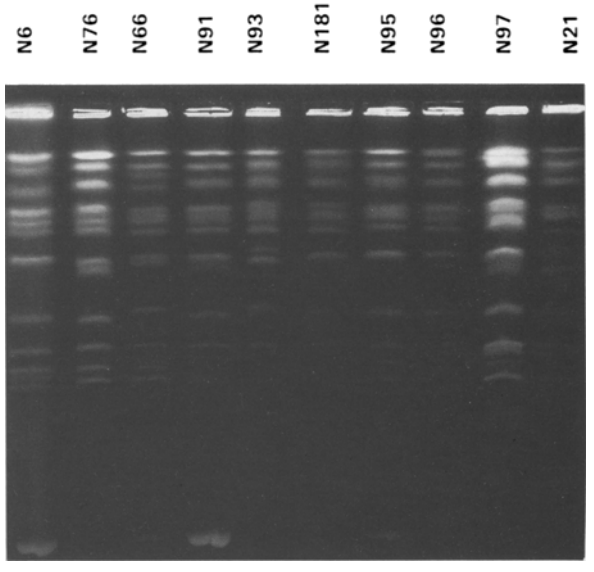

Fig. 3. Contour clamped homogeneous electric field (CHEF) banding patterns of chromosomal DNA of wine yeast strains N6, N76, N66, N91, N93, N181, N95, N96, N97 and N21. Intact chromosomal DNAs were separated in a 1.2% agarose gel stained with ethidium bromide.

profiles were obtained for strains N6 and N76, their DNA restriction fragment and chromosomal banding patterns were different.

Hybridisation of wine yeast strains

The aim of this breeding programme (Fig. 4) was to obtain hybrids that contain a combination of the oenological characteristics of strains N96 and N181, and of strains N76 and N96, respectively. Strain N96 is a K_2 killer (Kil$^+$) and is unable to utilize galactose as carbon source (Gal$^-$), whereas strains N76 and N181 are sensitive for the K_2 killer toxin (Kil$^-$) and capable of galactose assimilation (Gal$^+$). A haploid was isolated from the heterothallic strain N96 and was designated H96^H. No stable haploids could be isolated from the homothallic strains N76 and N181. Ascospores of strains N76 and N181 were therefore mixed with cells of haploid strain N96^H using the mass spore-cell mating procedure. This resulted in the formation of diploids, albeit at low frequency. The diploids were isolated by screening for both killer activity and galactose utilisation. One hybrid strain, USM30, resulted from the genetic cross between strains N96^H and N181. Three hybrids, USM21, USM22 and USM23, were obtained from the mass spore-cell mating between strains N76 and N96^H. The hybrids (Kil$^+$ Gal$^+$) were compared to their parental strains (Kil$^+$ Gal$^-$ and Kil$^-$ Gal$^+$) by using protein profiles (Fig. 5), DNA restriction banding patterns (Fig. 6) and electrophoretic karyotypes (Fig. 7).

Discussion

Traditional methods for distinguishing wine yeast strains have depended on morphological, physiological and biochemical criteria (Kunkee & Amerine 1970). These taxonomic procedures allow for distinction between species, but are time consum-

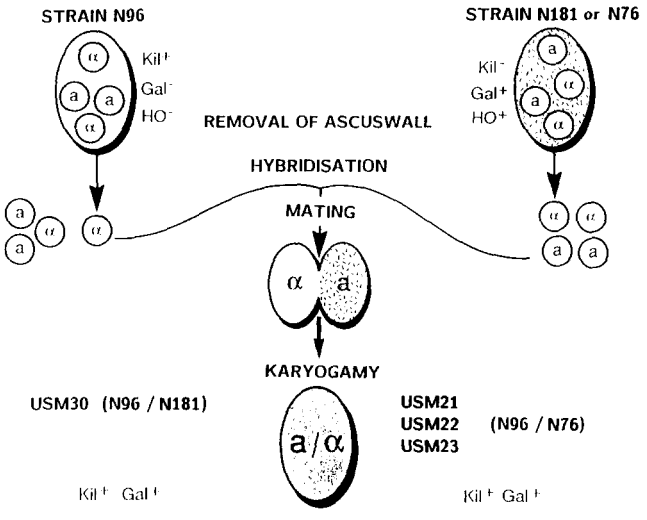

Fig. 4. The breeding strategy scheme. A haploid, N96^{H}, isolated from a heterothallic strain, N96, was hybridised with two homothallic strains, N76 and N181, by mass spore-cell mating. Strain N96 is a killer yeast (Kil$^+$) and is unable to utilise galactose as carbon source (Gal$^-$), whereas strains N76 and N181 are sensitive for the K_2 killer toxin (Kil$^-$) and capable of galactose assimilation. Hybrid USM30 (Kil$^+$ Gal$^+$) was obtained from the genetic cross between N96^{H} and N181, and three hybrid strains USM21, USM22 and USM23 (Kil$^+$ Gal$^+$) were obtained from the cross between N76 and N96.

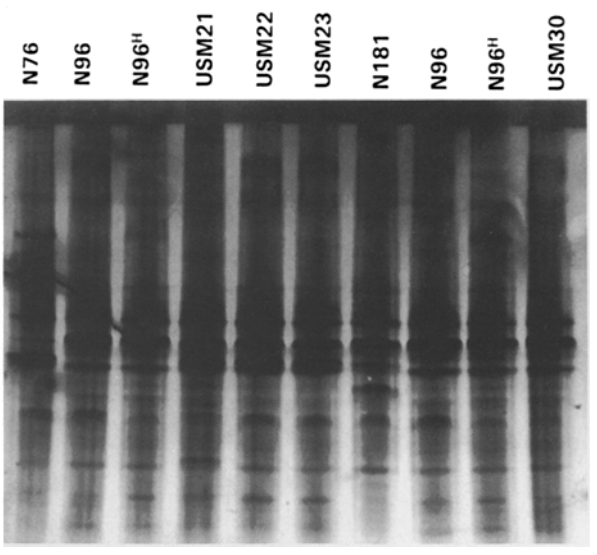

Fig. 5. Total soluble cell protein profiles of parental strains N76, N96^{H} and N181 and hybrid strains USM21, USM22, USM23 and USM30. Electrophoresis was performed in a 5% (w/v) acrylamide stacking gel and a 7% (w/v) acrylamide resolving gel.

ing and not always reliable. New approaches attempt to identify yeast strains by an analysis of their protein and DNA content (Meaden 1990).

Numerical analysis of total soluble cell proteins has been used to fingerprint and group wine yeasts (Van Vuuren & Van der Meer 1987) and brewing yeasts (Van Vuuren & Van der Meer 1988). Since a number of reputedly genetically unique yeast strains are being sold commercially, it has become necessary to fingerprint individual yeast strains used in wine fermentations. Van Vuuren & Van der Meer (1987) concluded that visual comparison of total soluble cell protein patterns can be used to fulfil this need in the wine industry. Our results confirmed this statement. Unique protein profiles were obtained for strains N6, N21, N66, N76, N95 and N97 (Fig. 1). Furthermore, we found that protein profiles could also be used to differentiate hybrid and parental strains in a breeding programme. The protein profiles of hybrid USM30 and its parental strains, N96^{H} and N181, were similar (Fig. 5). This was also evident in the cross between strains N76 and N96^{H}. The protein banding patterns of hybrids USM21, USM22 and USM23 were similar and contained a combination of the prominent unique bands present in the profiles of parental strains N76 and N96^{H} (Fig. 5).

Direct analysis of restriction fragments of mitochondrial and genomic DNA, using a number of restriction endonucleases, has been applied to differentiate brewing strains, but with mixed success. Aigle et al. (1984) found that restriction fragment banding patterns obtained from the mitochondrial DNA (mtDNA) of different lager strains were identical. Martens et al. (1985) could distinguish between the mtDNA of two ale strains, using double digestion with *Hin*dII and *Hin*dIII. Lee & Knudsen (1985) reported slight (but nevertheless discrete) differences in the pattern of *Ava*I or *Hae*II mtDNA restriction fragments of two lager strains. The preparation of genomic DNA is much more rapid and technically less demanding than the isolation of mtDNA. However, the interpretation of electrophoretic banding patterns of genomic DNA restiction fragments is complicated because discrete fragments are generally not apparent un-

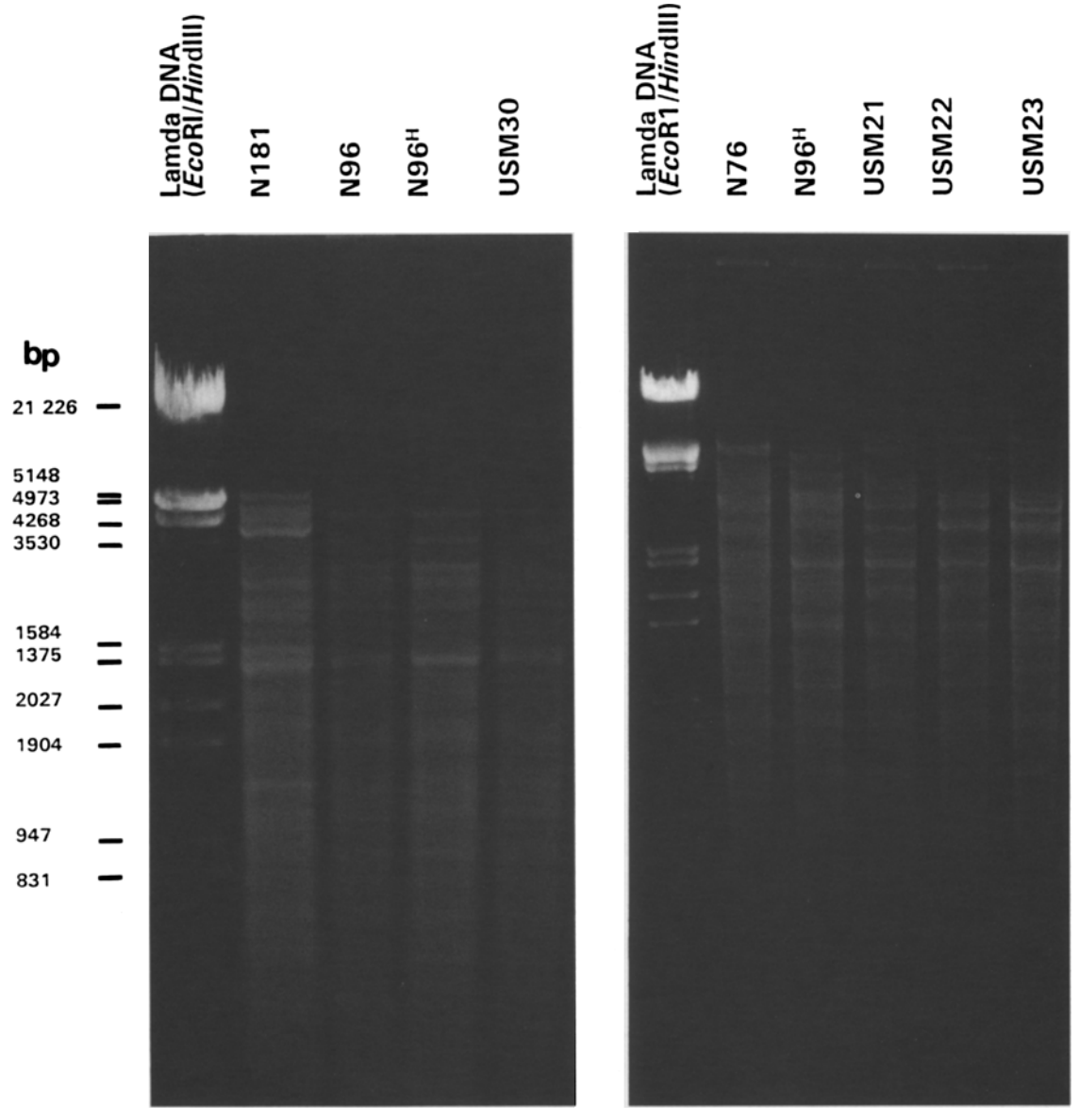

Fig. 6. DNA restriction fragment banding patterns of parental strains N76, N96^{H} and N181 and hybrid strains USM21, USM22, USM23 and USM30. Total genomic DNA of these strains was cleaved with *Hae*III and separated in a 0.8% agarose gel, stained with ethidium bromide.

less they are derived from repeated sequences such as ribosomal DNA (Meaden 1990). Pedersen (1985b) found that the *Eco*RI digested fragments of genomic DNA isolated from 22 Bavarian lager and ale strains generated identical electrophoretic banding patterns. Minor differences in the electrophoretic banding patterns of *Hpa*I digested DNA restriction fragments of ale and lager strains were reported by Panchal et al. (1987). From these results Meaden (1990) concluded that direct analysis of DNA restriction fragments was limited in the information it can provide and that it was therefore unlikely to be a useful method for fingerprinting large number of different brewing strains. By contrast our results indicated that direct analysis of DNA restriction fragments was a valuable tool to

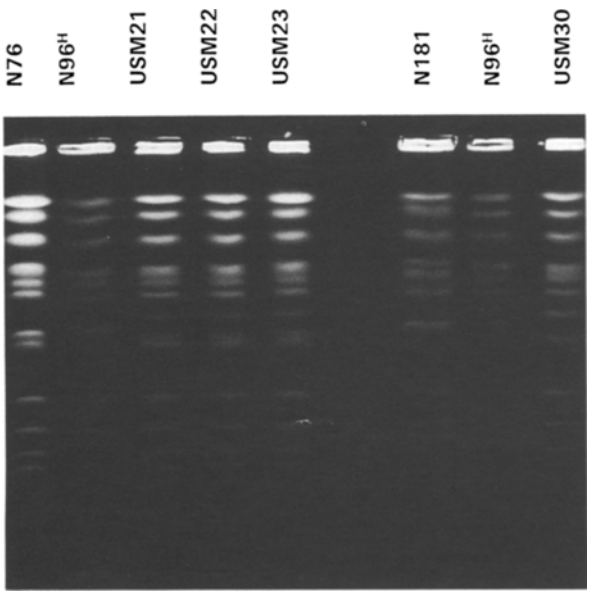

Fig. 7. Contour clamped homogenous electric field (CHEF) banding pattens of chromosomal DNA of parental strains N76, $N96^H$ and N181 and hybrid strains USM21, USM22, USM23 and USM30. Intact chromosomal DNAs were separated in a 1.2% agarose gel stained with ethidium bromide.

fingerprint wine yeast strains and to differentiate hybrid and parental strains in a breeding programme. The electrophoretic banding patterns of *Hae*III digested DNA restriction fragments of strains N6, N21, N66, N76, N95 and N97 were unique (Fig. 2). The DNA restriction banding patterns of hybrid USM30 and its parental strains, $N96^H$ and N181, were different (Fig. 6). The DNA restricton fragment profiles of hybrids USM21, USM22 and USM23 contained only slight variations, whereas their profiles were quite different from those of their parental strains, N76 and $N96^H$. In fact, this rapid fingerprinting method was found to be efficient, rendering analysis of specific DNA restriction fragments by probing unnecessary. However, a substantial amount of DNA fingerprinting has been attempted using labelled DNA probes, including genes encoding rRNA (*RDN1*), enzymes of the pyrimidine (*URA3*) and amino acid synthetic (e.g., *HIS4, LEU2, TRP1*) and glycolytic pathways (*PDC1, PFK1, PFK2, PGI1, PGM1, PGK1, PYK1*) as well as transposable elements (*Ty*1) (Braus et al. 1985; Decock & Iserentant 1985; Keiding 1985; Laaser et al. 1989; Martens et al. 1985; Pedersen 1983a, b; Pedersen 1985a, b; Pedersen 1986a, b; Sakai et al. 1990; Seehaus et al. 1985). Probing for specific DNA fragments, however, has not taken full advantage of the wide range of cloned yeast genes that are available. Meaden (1990) concluded that any attempt to fingerprint yeast strains by DNA probing was best approached using a variety of probes and restriction endonucleases, until a combination that suits the investigator's needs was found. It can also be expected that molecular marking by integrating unique DNA oligonucleotides into the genomes of wine yeasts will eventually enable 'designer' fingerprinting. Gene amplification by the polymerase chain reaction (PCR) with subsequent sequencing, can also be anticipated to filter through to fingerprinting of wine yeast strains and genetic hybrids.

Since the first karyotyping of yeasts by pulsed field gradient electrophoresis (PFGE) (Schwartz & Cantor 1984) and orthogonal field alteration gel electrophoresis (OFAGE) (Carle & Olson 1985) researchers have applied pulsed field electrophoresis to fingerprint a diverse range of yeast genera and species (Casey et al. 1988; De Jonge et al. 1986; Johnston & Mortimer 1986; Johnston et al. 1988; Sor & Fukuhara 1989; Takata et al. 1989). However, rather little has been published on the use of electrophoretic karyotyping to specifically differentiate wine yeast strains. Petering et al. (1988) differentiated several wine yeast strains by transverse alternating field electrophoresis (TAFE). Vezinhet et al. (1990) have reported 20 different TAFE karyotypes for 22 wine yeast strains. Only three strains originating from the same vinyard could not be differentiated by TAFE karyotyping. Our results showed eight different CHEF karyotypes for ten wine yeast strains (Fig. 3). Two strains (N96 and N181) originated from the same culture and strain N91 is a derivative from N96, cured of the K_2 killer MdsRNA. We have also shown that CHEF karyotyping was valuable in the analysis of genetic hybrids in breeding programmes (Fig. 7). The electrophoretic karyotype of hybrid USM30 differed from those of its parental strains, $N96^H$ and N181. The chromosomal banding patterns of hybrids USM21, USM22 and USM23 were identical

but differed from those of their parental strains, N76 and N96^{H}. Casey & Pringle (1990) reported that chromosome profiles could also be used in selecting variants with enhanced fermentation performance. In addition, gels with chromosomal banding patterns can also be blotted onto filters and probed with specific DNA fragments to differentiate between various yeasts (Hansen et al. 1990; Pretoruis & Marmur 1988; Takata et al. 1989). In this study, CHEF karyotyping without chromoblotting was sufficient to differentiate between the parental and hybrid strains.

In conclusion, results obtained by electrophoretic protein and DNA fingerprinting and karyotyping were found to be reproducible and valuable in the identification and control of industrial wine yeasts. Furthermore, these techniques enabled us to select four hybrid strains after mass spore-cell mating of wine yeasts. These hybrids fermented efficiently and produced wines with desirable oenological characteristics (Van Wyk & Pretorius 1990).

Acknowledgements

We thank Dr. Leon Dicks and Bernard Janse for their comments on the manuscript. We also thank the KWSI for financial support.

References

Aigle M, Erbs D & Moll M (1983) Determination of brewing yeast ploidy by DNA measurement. J. Inst. Brew. 89: 72–74

Augustyn OPH & Kock JLF (1989) Differentiation of yeast species, and strains within a species, by cellular fatty acid analysis. 1. Application of an adopted technique to differentiate between strains of *Saccharomyces cerevisiae*. J. Microbiol. Meth. 10: 9–23

Braus G, Furter R, Prantl F, Niederberger P & Hütter R (1985) Arrangement of genes *TRP1* and *TRP3* of *Saccharomyces cerevisiae* strains. Arch. Microbiol. 142: 383–388

Carle GF & Oslon MV (1985) An electrophoretic karyotype for yeast. Proc. Natl. Acad. Sci. USA 82: 3756–3760

Casey GP, Xiao W & Rank GH (1988) Application of pulsed field chromosome electrophoresis in the study of chromosome XIII and the electrophoretic karyotype of industrial strains of *Saccharomyces* yeasts. J. Inst. Brew. 94: 239–243

Casey GP & Pringle AT (1990) The relationship between yeast karyotype and fermentation performance. Eur. Brew. Conv. Proc. Cong. 23rd, Copenhagen (pp 1–9)

Decock JP & Iserentant D (1985) Ty hybridisation patterns of yeast and their use for strain identification. Eur. Brew. Conv. Proc. Cong. 20th, Helsinki (pp 195–202)

De Jonge P, Jonge FCM de, Meijers R, Steensma HY & Scheffers WA (1986) Orthogonal-field-altenation gel electrophoresis banding patterns of DNA from yeasts. Yeast 2: 193–204

Gupta NJ & Jones KW (1987) A DNA sequence which shows genomic variation in *a*, alpha and *HO* strains of *Saccharomyces cerevisiae*. Curr. Genet. 12: 161–166

Hansen M, Röcken W & Emeis C-C (1990) Construction of yeast strains for the production of low-carbohydrate beer. J. Inst. Brew. 96: 125–129

Johnston JR, Contopoulou CR & Mortimer RK (1989) Karyotyping of yeast strains of several genera by field inversion gel electrophoresis. Yeast 4: 191–198

Johnston JR & Mortimer RK (1986) Electrophoretic karyotyping of laboratory and commercial strains of *Saccharomyces* and other yeasts. Int. J. Syst. Bacteriol. 36: 569–572

Keiding AK (1985) Genetic and molecular characterization of a distiller's yeast. Carlsberg Res. Commun. 50: 95–125

Kunkee RE & Amerine MA (1970) Yeast in wine-making. In: Harrison JS & Rose AH (Eds). The Yeast, Vol III (pp 5–57) Academic Press, New York

Laaser G, Möller E, Janke K-D, Bahnweg G, Prillinger H & Prell HH (1989) Ribosomal DNA restriction fragment analysis as a taxonomic tool in separating physiologically similar basidiomycetous yeasts. System. Appl. Microbiol. 11: 170–175

Lee SY & Knudsen FB (1985) Differentiation of brewery yeast strains by restriction endonuclease analysis of their mitochondrial DNA. J. Inst. Brew. 91: 169–173

Martens FB, Berg R van den & Harteveld PA (1985) DNA fingerprinting of yeast strains. Eur. Brew. Conv. Proc. Cong. 20th, Helsinki (pp 211–217)

Meaden P (1990) DNA fingerprinting of brewers' yeast: current perspectives. J. Inst. Brew. 96: 195–200

Panchal CJ, Bast L, Dowhanick T & Stewart GG (1987) A rapid, simple and reliable method of differentiating brewing yeast strains based on DNA restriction patterns. J. Inst. Brew. 93: 325–327

Pedersen MB (1983a) DNA sequence polymorphisms in the genus *Saccharomyces*. I. Comparison of the *HIS4* and ribosomal RNA genes in lager strains, ale strains and various species. Carlsberg Res. Commun. 48: 485–503

— (1983b) Fingerprinting of DNA in the identification of yeast. Eur. Brew. Conv. Proc. Cong. 19th, London (pp 457–463)

— (1985a) Brewing yeast identification by DNA fingerprinting. Eur. Brew. Conv. Proc. Cong. 20th, Helsinki (pp 202–210)

— (1985b) DNA sequence polymorphisms in the genus *Saccharomyces*. II. Analysis of the genus *RDN1*, *HIS4*, *LEU2* and *Ty* transposable elements in Carlsberg, Tuborg and 22 Bavarian brewing strains. Carlsberg Res. Commun. 50: 263–272

— (1986a) DNA sequence polymorphisms in the genus *Saccharomyces*. III. Restriction endonuclease fragment patterns of

chromosomal regions in brewing and other yeast strains. Carlsberg Res. Commun. 51: 163–183

— (1986b) DNA sequence polymorphisms in the genus *Saccharomyces*. IV. Homologous chromosomes III of *Saccharomyces bayanus*, *S. carlsbergensis* and *S. uvarum*. Carlsberg Res. Commun. 51: 185–202

Petering J, Langridge P & Henschke P (1988) Fingerprinting wine yeast. Aust. N.Z. Wine Ind. J. 3: 48–52

— (1990) Identification of yeast strains by molecular biology techniques. International Association for Modern Winery Technology and Management e.V. Oenol. Symp. 9th, Cascais

Plummer DT (1971) An Introduction to Practical Biochemistry. McGraw-Hill Book Co. Ltd., London

Pretorius IS & Marmur J (1988) Localization of yeast glucoamylase genes by PFGE and OFAGE. Curr. Genet. 14: 9–13

Pretorius IS & Westhuizen TJ van der (1991) Impact of yeast genetics and recombinant DNA technology on the wine industry. S. Afr. J. Enol. Vitic. 12: 3–31

Sakai A, Shimizu Y & Hishinuma F (1990) Integration of heterologous genes into the chromosome of *Saccharomyces cerevisiae* using a delta sequence of yeast retrotransposon Ty. Appl. Microbiol. Biotechnol. 33: 302–306

Salmon J-M, Pinon R & Gancedo C (1989) Isolation and characterization of mutants of *Saccharomyces cerevisiae* able to sporulate in the presence of glucose. J. Gen. Microbiol. 135: 203–209

Schwartz DC & Cantor CR (1984) Separation of chromosomal-sized DNAs pulsed field gradient gel electrophoresis. Cell 37: 67–75

Seehaus T, Rodicio R, Heinisch J, Aguilera A, Schmitt HD & Zimmerman FK (1985) Specific gene probes as tools in yeast taxonomy. Curr. Genet. 10: 103–110

Sherman F, Fink GR & Hicks JB (1986) Laboratory Course Manual for Methods in Yeast Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Sor F & Fukuhara H (1989) Analysis of chromosomal DNA patterns of the genus *Kluyveromyces*. Yeast 5: 1–10

Takata Y, Watari J, Nishikawa N & Kamada K (1990) Electrophoretic banding patterns of chromosomal DNA from yeasts. Am. Soc. Brew. Chem. 47: 109–113

Thornton RJ (1983) New yeast strains from old- the application of genetics to wine yeasts. Food Technol. Australia 35: 46–50

Tredoux HG, Kock JLF, Lategan PM & Muller HB (1987) A rapid identification technique to differentiate between *Saccharomyces cerevisiae* strains and other yeast species in the wine industry. Am. J. Enol. Vitic. 38: 161–164

Van der Westhuizen TJ & Pretorius IS (1989) Genetic characterization and breeding of wine yeasts. S. Afr. Soc. Enol. Vitic. Cong. 13th, Cape Town

— (1990) Genetic characterization and breeding of wine yeasts. S. Afr. Soc. Microbiol. Cong. 6th, Stellenbosch (pp 15.15)

Van Vuuren HJJ & Meer LJ van der (1987) Fingerprinting of yeasts by protein electrophoresis. Am. J. Enol. Vitic. 38: 49–53

— (1988) Characterization of brewing yeast strains by numerical analysis of total soluble cell protein patterns. S. Afr. J. Enol. Vitic. 94: 245–248

Van Wyk CJ & Pretorius IS (1990) A comparative study of new yeast hybrids. S. Afr. Soc. Enol. Vitic. Cong. 14th, Cape Town

Vezinhet F, Blondin B & Hallet J-N (1990) Chromosomal DNA patterns and mitochondrial DNA polymorphisms as tools for identification of enological strains of *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 32: 568–571